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Sequestration of Inhaled Particulate Antigens by Lung Phagocytes

A Mechanism for the Effective Inhibition of Pulmonary Cell-Mediated Immunity

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Dendritic cells (DCs) have emerged as the dominant antigen-presenting cells (APCs) of the lung, playing a vital role in the induction of cell-mediated immunity to inhaled antigens. We have previously demonstrated that an airway challenge with the soluble antigen hen egg lysozyme yields rapid acquisition of specific antigen-presenting cell activity by purified pulmonary DCs and a cell-mediated immune response in the lung upon secondary challenge. To examine how a particulate antigen leads to a cell-mediated response* in vivo*, graded concentrations of heat-killed* Listeria *(HKL) were injected intratracheally into Lewis rats. The bacteria were rapidly ingested by lung macrophages and polymorphonuclear leukocytes. The ability of purified pulmonary DCs pulsed* in vivo *by an airway challenge with HKL to subsequently stimulate HKL-specific responses* ex vivo *showed a threshold response, requiring a dose in excess of 10^9 organisms/rat. By contrast, all dosages of HKL yielded specific sensitization of lymphocytes in the draining hilar nodes. Pulmonary DCs purified from rats after a secondary* in vivo *airway challenge with HKL at day 14 were ineffective antigen-presenting cells except at high dosages of antigen. The generation of cell-mediated pulmonary inflammation paralleled the antigen-presenting cell activity of pulmonary DCs and was observed only at high antigen dosages. Hen egg lysozyme immobilized onto polystyrene beads and injected intratracheally yielded comparable results to those observed with HKL. We suggest that a pulmonary cellular immune response is generated to an inhaled particulate antigen when the protective phagocytic capacities of the lung are exceeded and antigen is able to interact directly with interstitial DCs. The diversion of particulate antigens by pulmonary phagocytes may help to limit undesirable pulmonary inflammation while allowing the generation of antigen-specific immune lymphocytes* in vivo. *(Am J Pathol 1996, 148:657–666)

Dendritic cells (DCs)[1] are widely distributed in the pulmonary airways and interstitium.[1–5] DCs have emerged as the dominant antigen-presenting cells (APCs) of the lung, where they serve a critical role in the induction of cell-mediated immunity.[3,6–9] Immature DCs, which resemble Langerhans cells by virtue of their expression of high levels of class II major histocompatibility complex (Ia) antigen, surface FcR, and their ability to ingest particulates,[10–12] reside principally in the pulmonary airways and may transport inhaled antigen to local draining lymph nodes (LNs).[13–15] By contrast, mature Ia$^+$ connective tissue DCs are located primarily in the collagenous interstitium of the lung where they are distinguished by their absence of FcR, their limited ingestion of particulates, and their efficiency in presenting antigens to naive and sensitized T cells[5,12,16–18]

The role of pulmonary DCs in the presentation of inhaled particulate antigens remains poorly defined. After inhalation, particulate antigens are rapidly in-

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gested by pulmonary phagocytes including alveolar macrophages (MØs). Although lung MØs can transport antigens to local LNs,[19–21] they display limited capacities to present antigens to T cells effectively.[22,23] In addition, MØs do not appear to cooperate in antigen presentation by generating particulate antigen peptides for presentation by DCs.[24,25] Most studies suggest that pulmonary MØs actively antagonize the APC activities of DCs.[7,8,18,26]

The ability of DCs to phagocytize particulate antigens has been controversial.[27,28] Reis e Sousa et al[27] have demonstrated the phagocytosis of a variety of particulate antigens by Langerhans cells *in vitro*. Inaba et al[28] have demonstrated that DC progenitors internalize particulate antigens (*Bacillus* Calmette-Gerin) *in vitro* and retain immunogenic peptides, as they mature, for presentation to T cells. Furthermore, DCs that are pulsed with particulate antigen *in vitro* and subsequently injected *in vivo* can sensitize naive T cells.[28]

In the present study, we examined the interaction of pulmonary phagocytes and DCs during an airway response to the particulate antigen heat-killed *Listeria* (HKL) *in vivo*. Our results demonstrate that alveolar MØs and polymorphonuclear leukocytes (PMNs) effectively sequester particulate antigens away from pulmonary interstitial DCs without compromising the sensitization of antigen-specific T cells in the draining LN.

Materials and Methods

Animals

Inbred, pathogen-free, 6- to 8-week-old female Lewis rats (150 to 250 grams) were obtained from Charles River Laboratories (Kingston, MA). Rats were housed in a restricted access animal care facility and permitted access to food and water *ad libitum*.

Reagents and Antibodies

Heat-killed Listeria *monocytogenes*

HKL were obtained from the Massachusetts General Hospital Bacteriology Laboratory. The concentration of organisms was determined by McFarland standards and the bacteria were heat-killed in a 63°C water bath for 90 minutes. Viability was assessed by the failure of the bacteria to grow on blood agar plates. Aliquots (10^9 bacteria/ml in saline) were stored at −20°C.

Hen Egg Lysozyme (HEL)

HEL (Sigma Chemical Co., St. Louis, MO) was prepared at a concentration of 1 mg/ml in normal saline, filtered through a 0.45-μm low protein binding filter (Acrodisc 4184, Fisher Scientific, Boston, MA) and stored at 4°C.

Fluorescein-Labeled HKL

HKL were fluorescein labeled by a modification of the protocol of Drevets and Campbell.[29] HKL (10^{10} organisms) were washed in phosphate-buffered saline (PBS) and resuspended in 1 ml of 0.1 mol/L sodium bicarbonate, pH 9.0, with 50 μl of fluorescein isothiocyanate (Sigma; 0.05 mg/ml in 0.1 mol/L sodium bicarbonate, pH 9.0) and incubated for 4 hours at room temperature with gentle end-to-end mixing. The fluorescein-labeled HKL were washed free of unbound fluorochrome and stored frozen at −20°C before use.

Chloral Hydrate

Chloral hydrate (Fisher Scientific) was administered as an anesthetic at a dose of 400 mg/kg.

Enzymatic Digests

Collagenase (150 U/ml; Worthington Biochemical Corp., Freehold, NJ) and DNAse (50 U/ml; Sigma) were freshly prepared for digestion of rat lung.

Bovine Serum Albumin (BSA)

BSA was prepared by a modification of the procedure of Steinman and Cohn.[30] Briefly, 10.5 g of BSA powder, fraction V (Intergen, Purchase, NY), was solubilized in 2.9 ml of 1 N NaOH, 5.8 ml of double-distilled water, and 18.6 ml of PBS to yield a final pH of 7.35 ± 0.05 and a density of 1.080, as judged by density refractometry (ABBE-3L, 33–46-10; VWR Scientific, Boston, MA). The solution was filtered with a 0.45-μm filter with prefilter (Nalgene, Fisher Scientific) and maintained at 4°C before use.

Complete Medium (CM) and Culture Conditions

Cells were cultured in RPMI 1640 (JRH Biosciences, Lenexa, KS), 10% heat-inactivated fetal bovine serum (Sigma), 50 μg/ml gentamycin (GIBCO BRL, Gaithersburg, MD), 0.5% 1 mol/L HEPES Buffer (GIBCO BRL), and 5×10^{-5} mol/L 2-mercaptoetha-

nol (Sigma), and incubated at 37°C in a humidified chamber of 95% air and 5% CO_2.

[^{3}H]TdR

Radiolabel was used at 1 μCi/well (specific activity 81.0 Ci/mmol; DuPont/New England Nuclear, Boston, MA).

Monoclonal Mouse Anti-Rat Antibodies

Anti-rat monoclonal antibodies were used to purify and characterize cells in these studies. These included W3/13 (CD43), W3/25 (CD4), OX-8 (CD8), OX-39 (CD25), OX-1 (CD45R), OX-22 (CD45RC), OX-43, OX-6 (Ia), (all from Accurate Chemical & Scientific Co., Westbury, NY); ED1 and ED2 (generous gift of C. Dijkstra), anti-IgM, OX-12 (anti-κ light chain), and OX-33 (CD45RA) (all from Pharmingen, San Diego, CA). The monoclonal antibodies were produced either as ascites or supernatants and were used at predetermined optimal concentrations. For *in situ* localization, purified DCs or lung tissues were stained by an avidin-biotin immunoperoxidase technique, as previously described.[15] The surface immune phenotype of cells in suspension was examined in a Becton-Dickinson FACScan cytofluorimeter after either direct staining with fluorescein-conjugated anti-rat monoclonal antibodies or indirect staining with goat $F(ab')_2$ anti-mouse IgG-fluorescein isothiocyanate (Biosource International, Camarillo, CA).[31]

Coupling of Soluble Antigens to Polystyrene Beads

Antigens were covalently linked to polystyrene beads using the directions supplied by the manufacturer (Polysciences, Warrington, PA). In brief, 1 ml of a 2.5% suspension of beads (3 μm diameter) were transferred to an Eppendorf tube and washed three times in PBS. The washed beads were resuspended in 1 ml of 8% glutaraldehyde in PBS, pH 7.4, and were left overnight at room temperature with gentle end-to-end mixing. The beads were washed three times with PBS before resuspension with 500 μg of protein in PBS, incubated for 4 to 5 hours at room temperature with gentle end-to-end mixing, and centrifuged for 10 minutes; the supernatant was saved for protein determination. Beads were resuspended in 1 ml of 0.5 mol/L ethanolamine in PBS, centrifuged and resuspended in 1 ml of 10 mg/ml BSA in PBS for 30 minutes, and stored in storage buffer (10 mg/ml BSA, 0.1% NaN_3, and 5% glycerol). The beads were washed three times in PBS and counted in a hemocytometer before *in vivo* use.

Generation of HEL- and HKL-Immune T Cells

Rats were immunized with an emulsion of 100 μg of HEL or 1×10^7 HKL in complete Freunds' adjuvant (Difco Laboratories, Detroit, MI). The emulsion (0.1 ml) was injected bilaterally at the base of the tail; inguinal LNs were harvested at 10 to 14 days, mechanically dispersed, and separated on Isolymph (Gallard Schlesinger Industries, Carle Place, NY). LN mononuclear cells (2×10^6) were incubated in 24-well culture plates with HEL (100 μg/ml) or HKL (10^7/ml) antigen in a humidified chamber of 95% air and 5% CO_2. Recombinant human interleukin-2 (100 U/ml; Cetus Corp., Emeryville, CA) was added to the medium at day 5 and then two to three times weekly. Every 3 to 4 weeks, the cultures were restimulated with HEL or HKL, respectively, in the presence of normal irradiated (3000 cGy) syngeneic spleen cells at a ratio of 10:1 spleen cells to T-cell blasts. Immune phenotyping by cytofluorimetry showed the antigen-specific lymphoblasts were >95% W3/25$^+$ (CD4) OX22$^-$ (CD45RC). The specificity of the response was judged by a greater than threefold difference in the magnitude of [^{3}H]TdR incorporation by T-cell blasts to the immunogen compared with an irrelevant antigen.

Intratracheal (i.t.) Instillation of Antigen

Rats were lightly anesthetized with chloral hydrate (400 mg/kg), and the trachea was surgically exposed. Equal volumes (0.1 ml) of the test antigen (eg, HKL, soluble HEL, immobilized HEL, or immobilized BSA), normal saline, and indocyanine green (2.5 mg/ml) were introduced into the trachea slowly via a 25-gauge needle. The rats were subsequently sacrificed at the times indicated in Results. In some experiments, rats were rechallenged with HKL i.t. on day 14 and sacrificed at 48 hours. Rats receiving antigen-coated beads were anesthetized as above and 0.3 ml of a suspension of beads (2.5×10^7 beads/ml) was instilled per rat (approximately 50 μg protein/rat).

DC Purification

DCs were purified from lung as previously described.[4] Briefly, lungs were perfused with saline via the pulmonary artery to diminish blood contaminants. Excised lung tissue was digested with collagenase and DNAse, fractionated by BSA density gradient sedimentation,

and the desired mononuclear cell fraction was adhered on tissue culture dishes (3003/Falcon, Fisher Scientific) with CM for 2 hours at 37°C. Nonadherent cells were discarded and the adherent fraction was cultured overnight at 37°C. Loosely adherent cells enriched for DCs were further subjected to immunopanning with OX-6. The selected cells were retrieved by gentle scraping with a rubber policeman and applied to plastic tissue culture dishes for 1 hour at 37°C to remove adherent mϕ. The nonadherent cells were judged to be highly enriched (>90%) for DCs based on their morphology, failure to stain for nonspecific esterase, immunostaining characteristics, and ability to stimulate a primary allogeneic mixed lymphocyte reaction.[4]

Bronchoalveolar Lavage (BAL)

BAL was carried out as previously described.[32] Briefly, animals were anesthetized with chloral hydrate and the trachea was surgically exposed and cannulated with plastic tubing. BAL was performed with 5 ml of PBS, pH 7.3, containing 0.6 mmol/L EDTA. Lavage fluid was left in the lung for 1 minute while chest massage was performed. BAL was repeated six times for each animal.

Accessory Cell Activities

DCs from the lung and LN were irradiated (3000 cGy), suspended in CM, and plated (1×10^4 cells/well) into 96-well flat-bottom culture plates (Falcon, Fisher Scientific). Nylon-wool-treated and OX-6^+-cell-depleted splenic lymphocytes, HEL-immune, or HKL-immune T cells (5×10^4) were added to DCs with or without HEL (100 μg/ml) or HKL (10^7 to 10^{10} bacteria/ml). The culture plates were incubated for 72 hours at 37°C, and the wells were pulsed with [^{3}H]TdR (1 μCi/well) for 6 hours before harvesting. The pulsed wells were harvested in a semi-automatic cell harvester (Skatron, Sterling, VA) and counted in a Tri-Carb liquid β-scintillation spectrometer (Packard Instrument Co., Sterling, VA). The percent relative response was calculated by the formula previously described:[15]

$$\left\{ \text{cpm} \times \frac{(\text{immune T cells} + \text{Ag-pulsed DCs})}{(\text{immune T cells} + \text{Ag-pulsed DCs} + \text{Ag})} - \text{cpm} \times \frac{(\text{immune T cells} + \text{control DCs})}{(\text{immune T cells} + \text{control DCs} + \text{Ag})} \right\} \times 100$$

where the antigen (Ag) is either HKL or HEL and the immune T cells are either HKL- or HEL-immune T cells.

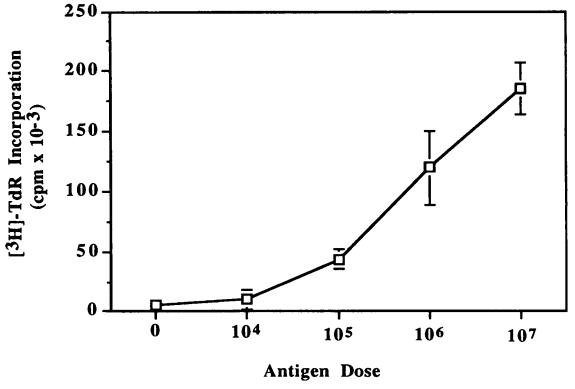

Figure 1. *APC activity of pulmonary DCs for HKL* in vitro. *DCs purified from the lungs of naive rats were able to support the specific proliferation of HKL-immune T cells* in vitro *in a dose-dependent manner. Data are the mean ± SD of triplicate samples from a representative experiment (three experiments performed).*

Lymphocyte Proliferation Assay

Hilar LN lymphocytes were suspended in CM and plated (1×10^5 cells/well) in triplicate into 96-well plates (Falcon). Irradiated (3000 cGy) splenic mononuclear cells were added (1×10^5 cells/well) as APCs. HKL was added at a concentration of 10^7 organisms/well; controls were plated with CM alone. The culture plates were incubated for 72 hours at 37°C, and the wells were pulsed with [^{3}H]TdR (1 μCi/well) for 6 hours before harvesting. The pulsed wells were harvested in a semi-automatic cell harvester (Skatron) and counted in a Tri-Carb liquid β-scintillation spectrometer (Packard).

Results

Pulmonary DCs Present Particulate Antigens to Primed T Cells in Vitro

Pulmonary DCs were greater than 95% OX-1^+ (CD45) and OX-6^+ (Ia^+); approximately 50% of the DCs in these preparations were FcR^+ and less than 5% stained for B cell, T cell, or MØ surface antigens, as previously described.[4,15] Pulmonary DCs were highly effective in presenting HKL to HKL-immune $CD4^+$ T cell lines in a dose-dependent response (Figure 1), confirming earlier observations that DCs are excellent APCs for HKL *in vitro*.[12,15]

Presentation of HKL by Pulmonary DCs after an in Vivo *Challenge*

Graded concentrations of HKL (10^7 to 10^{10} organisms/rat) were instilled i.t. into naive Lewis rats. Pulmonary DCs were subsequently purified from lung

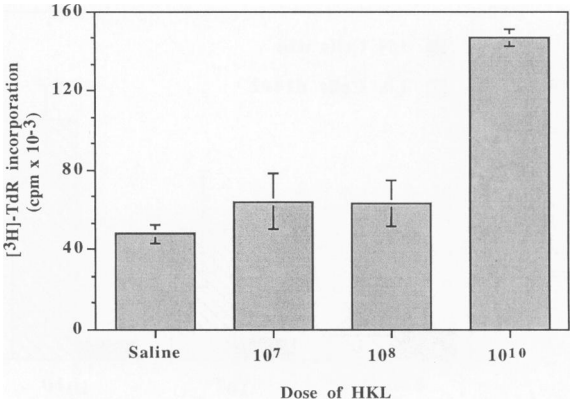

Figure 2. *Effect of antigen dose on APC activity of pulmonary DCs after HKL instillation i.t. APC activity of pulmonary DCs isolated from the lung 24 hours after introduction of HKL was dose dependent. At relatively low concentrations of HKL (10^7 to 10^8 organisms/rat), DCs were unable to stimulate [^{3}H]TdR incorporation into HKL-immune T cells, whereas rats receiving higher doses (10^{10} organisms/rat) showed increased APC activity compared with saline controls. Data are the mean ± SD of triplicate samples from a representative experiment (three experiments performed).*

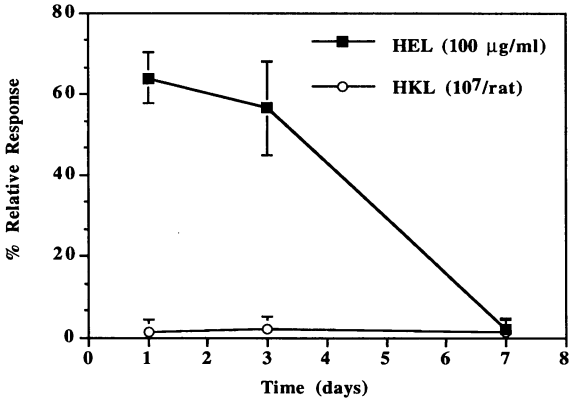

Figure 3. *Comparison of APC activity of pulmonary DCs after an i.t. challenge with either HEL or HKL. APC activity of pulmonary DCs isolated from lung was measured on days 1, 3, and 7 after instillation of either HEL or HKL i.t. HEL-challenged rats showed the rapid acquisition of excellent APC activity for HEL-immune T cells that diminished over the interval studied. In contrast, pulmonary DCs from rats receiving HKL (10^7/rat) showed no demonstrable APC activity. Data are expressed as the percent relative response ± SEM of three experiments.*

digests at 24 hours and examined for their ability to serve as APCs *in vitro* in the absence of supplementary HKL. Pulmonary DCs proved to be ineffective APCs at dosages of HKL between 10^7 and 10^9 organisms/rat (Figure 2). This was judged not to reflect an immunosuppressive effect in the culture, as supplementing these DCs with HKL (10^7/well) yielded lymphocyte proliferation that was at least five times greater than controls. Pulmonary DCs purified from rats that received 10^{10} HKL organisms i.t. showed good APC activity compared with controls in the absence of supplementary antigen (Figure 2).

To exclude the possibility that the poor APC activity of lung DCs was due to a delayed response in the lungs of rats receiving lower numbers of HKL, the APC activities of DCs purified at intervals up to 7 days after the HKL i.t. challenge were examined. In rats receiving HKL (10^7/rat), pulmonary DCs showed no demonstrable APC activity during the 7-day interval (Figure 3), whereas pulmonary DCs purified from rats treated in parallel with the soluble antigen HEL i.t. were excellent APCs (Figure 3), as previously reported.[15]

HKL Are Ingested by Pulmonary Phagocytes After an in Vivo *Pulmonary Challenge*

To ascertain the fate of HKL injected into the lung, graded numbers of unlabeled or fluorescein-labeled HKL (10^7 to 10^{10} organisms/rat) were instilled i.t. into naive rats. At 24 hours, the BAL fluid revealed a marked influx of PMNs (Figure 4). Gram-stained cytosmears from the fluid showed large numbers of gram-positive bacilli in both PMNs and MØs (not shown). The fluorescein-labeled HKL were seen in large numbers within MØs lining the alveolar septa, as judged by epifluorescence microscopy of frozen sections (Figure 5). At dosages of HKL greater than 10^9, small numbers of fluorescein-labeled HKL were detected within the alveolar interstitium (not shown). These observations suggest that HKL particles are rapidly ingested by phagocytes at the lung surface and that few bacteria enter the pulmonary interstitium at dosages less than 10^9 HKL/rat.

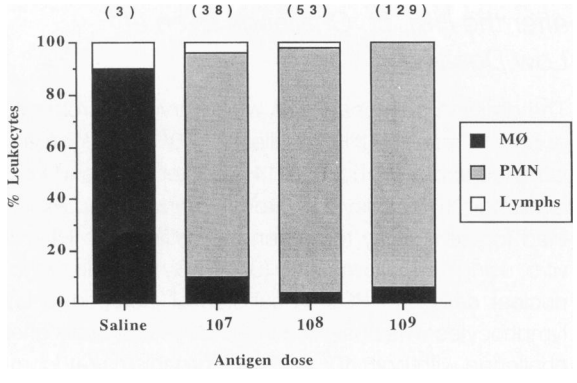

Figure 4. *Increase in phagocytic cells in the BAL of rats challenged with HKL i.t. Enumeration of leukocytes recovered from BAL 24 hours after i.t. challenge with graded concentrations of HKL demonstrated a dose-dependent increase in phagocytic cells compared with saline-challenged controls. Values in parentheses are the total cell number × 10^{-6} returned from the BAL. Data are from a representative experiment (five experiments performed).*

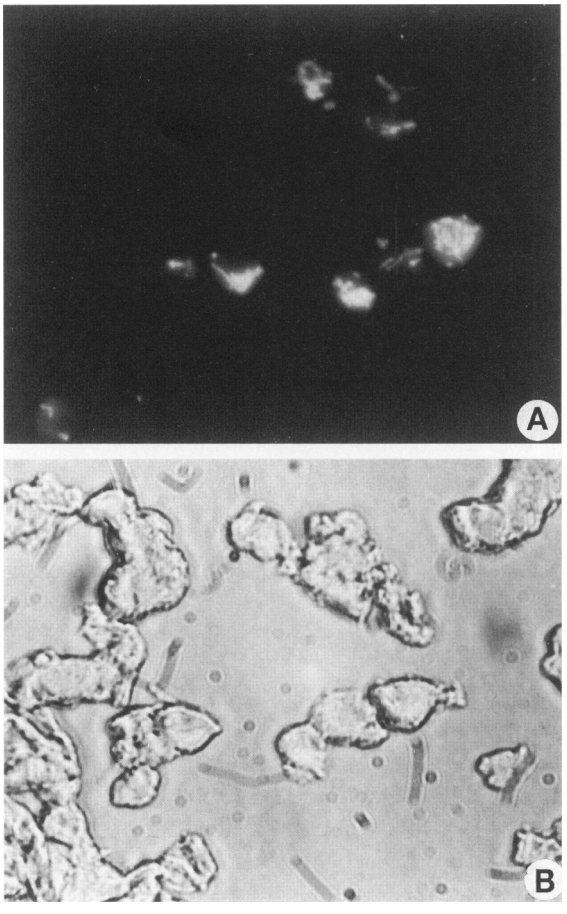

Figure 5. *Fluorescein-labeled HKL are ingested by phagocytes after* in vivo *pulmonary challenge.* **A:** *Rats challenged with fluorescein-labeled HKL (10^8/rat) show large numbers of intracellular bacteria within alveolar MØs along the alveolar septa.* **B** *Phase contrast appearance of field shown in* **A**. *Few neutrophils containing HKL were identified* in situ. *Magnification, ×400.*

T Cells in the Draining LN Are Sensitized after the HKL i.t. Challenge Even at Low Dosages

The draining pulmonary LNs were harvested from rats at day 14 after the HKL challenge (10^7 or 10^{10} organisms/rat) and at 48 hours (d14+2) after a second HKL challenge. Lymphocytes were enumerated and examined for their ability to respond specifically to HKL *in vitro*, using normal irradiated (3000 cGy) splenic mononuclear cells as APCs. A substantial increase in LN lymphocytes was observed in the d14+2 animals after challenge with both 10^7 and 10^{10} organisms/rat, compared with saline controls (Figure 6). LN cells harvested from day 14 rats displayed a proliferative response to HKL *in vitro* at both dosages of HKL, indicating that specific sensitization of LN T cells had effectively occurred *in vivo* (Table 1).

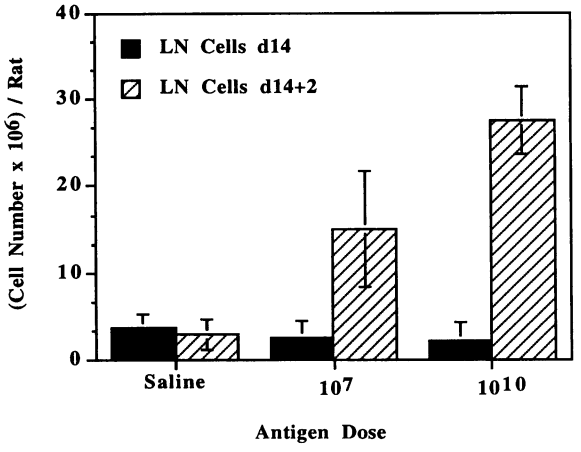

Figure 6. *Cell yield from hilar LN after HKL injection. Hilar LN cells were enumerated from rats on day 14 (d14) after primary challenge and 2 days after secondary challenge (d14+2) with HKL i.t. At day 14 there was no significant increase in LN cells compared with saline controls, whereas 2 days after secondary challenge there was a substantial increase in LN cells. Data are the mean ± SD from three experiments.*

Lung DCs Are Effective APC Only When High Dosages of Organisms Are Used in the Secondary HKL Challenge i.t.

In parallel, purified pulmonary DCs were examined for their ability to stimulate HKL-immune T cells after a secondary i.t. challenge with HKL (10^7 and 10^{10} organisms/rat; Table 2). Pulmonary DCs from rats challenged with 10^7 HKL i.t. showed little ability to serve as APCs for HKL-immune lymphocytes, whereas substantial APC activity was detected in the group challenged with 10^{10} organisms (Table 2). Addition of HKL to the culture wells resulted in substantial APC activity for both groups, demonstrating that the functional capacities of DCs were intact (Table 2).

Table 1. *Response of LN Lymphocytes to HKL after an* in Vivo *HKL Challenge*

In vivo condition	cpm × 10^{-3} ± SD	
Day 14	LNCs + medium	LNCs + HKL (10^7/well)
Saline i.t.	0.7 ± 0.1	2.0 ± 0.4
HKL (10^7/rat) i.t.	0.9 ± 0.2	10.9 ± 1.2
HKL (10^{10}/rat) i.t.	0.8 ± 0.2	8.6 ± 0.9

Hilar lymph node cells (LNCs) were harvested from rats 14 days after primary challenge with HKL i.t. (10^7 and 10^{10} organisms/rat). Proliferative response ([^{3}H]TdR incorporation) was assessed in the presence and absence of added HKL (10^7 organisms/well) using irradiated (3000 cGy) splenic mononuclear cells as APCs. Data are mean ± SD of triplicate samples from a representative experiment.

Table 2. *APC Activities of Pulmonary DCs after HKL Injection i.t.*

Dose of antigen	Secondary challenge	Additions to wells	Lung DC APC activity (cpm $\times 10^{-3}$) ± SD
Saline	None	None	0.2 ± 0.1
		HKL	61.4 ± 9.0
10^7	None	None	0.2 ± 0.1
		HKL	43.8 ± 7.0
10^7	2 days	None	1.5 ± 0.4
		HKL	15.0 ± 13
10^{10}	None	None	0.2 ± 0.1
		HKL	74.9 ± 12
10^{10}	2 days	None	5.8 ± 0.3
		HKL	114.6 ± 15

Pulmonary DCs were harvested from rats 14 days after primary challenge and 2 days after secondary challenge with HKL i.t. (10^7 and 10^{10} organisms/rat). APC activity was assessed by examining the ability of the DCs to support [^{3}H]TdR incorporation by HKL-immune T cells in the presence and absence of supplementary HKL (10^7/well).

Cell-Mediated Pulmonary Inflammation after a Secondary Challenge with HKL Is Dose Dependent

At day 14 after the primary HKL challenge the lungs showed essentially no inflammation. Rats receiving a second dose of 10^7 HKL showed a minimal increase in lung MØs and PMNs but little pulmonary lymphocytic inflammation at d14+2 (Figure 7A). However, rats that received a second challenge of 10^{10} HKL showed a marked perivenular pulmonary infiltrate that was composed primarily of lymphocytes, with smaller numbers of PMNs and MØs (Figure 7B). Controls that had not been sensitized to HKL showed only a modest increase in PMNs at 48 hours after the HKL challenge (not shown). Cytofluorimetric analysis of lymphocytes purified from lung digests of the rechallenged rats revealed a two- to threefold increase in CD3$^+$ T cells in the sensitized rats that received a secondary challenge of 10^{10} HKL compared with controls.

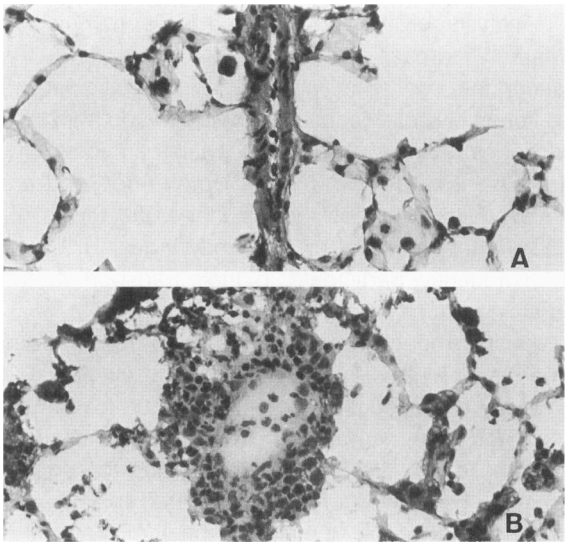

Figure 7. *Pulmonary inflammation after secondary challenge with 10^{10} HKL organisms/rat.* A: *HKL-sensitized rats challenged with 10^7 HKL showed little pulmonary inflammation.* B: *By contrast, sensitized rats that received the 10^{10} dosage of HKL i.t. on day 14 showed a prominent perivascular cuff of lymphoid cells with smaller numbers of PMNs and MØs. Increased interstitial PMNs were also present. Hematoxylin and eosin; magnification, ×300.*

The Injection of Immobilized HEL Fails to Elicit an APC Response by Pulmonary DCs

To determine whether the particulate nature of HKL was primarily responsible for our observations, soluble HEL was immobilized onto polystyrene beads and the ability of pulmonary DCs to present either soluble or immobilized HEL was compared *in vitro.* Pulmonary DCs proved to be highly effective APCs for both soluble HEL and immobilized HEL *in vitro* (Figure 8A).

Next, the ability of pulmonary DCs to serve as APCs for immobilized HEL after an *in vivo* challenge was examined. Naive rats were injected i.t. with (1) soluble HEL (10 μg/rat), (2) immobilized HEL (~10 μg/rat), or (3) immobilized BSA (~10 μg/rat). At 24 hours, pulmonary DCs from rats receiving soluble HEL yielded excellent proliferation of HEL-immune T cells, whereas pulmonary DCs from animals challenged with immobilized HEL or BSA did not (Figure 8B).

Examination of the BAL and lung showed that the HEL-beads injected i.t. had been ingested by pulmonary MØs and PMNs (Figure 9). When rats were sensitized with HEL-beads, no pulmonary cell-mediated immune response was generated after a second challenge with HEL-beads. However, rats sensitized with HEL-beads and subsequently challenged with soluble HEL (10 μg/rat) developed a perivenular lymphocytic infiltrate (not shown) that was comparable to that seen in controls that had been both sensitized and challenged with soluble HEL.[15]

Discussion

Specific cell-mediated immunity represents a critical pathway for limiting antigen penetration into the lung. In an earlier study, we demonstrated that the airway administration of a soluble antigen leads to specific APC activity by pulmonary DCs, sensitization of lymphocytes in the draining LNs, and a delayed perivascular cellular immune response in the lung upon secondary antigen challenge. In this report, we have examined the induction of cellular immunity to the particulate antigen HKL *in vivo*.

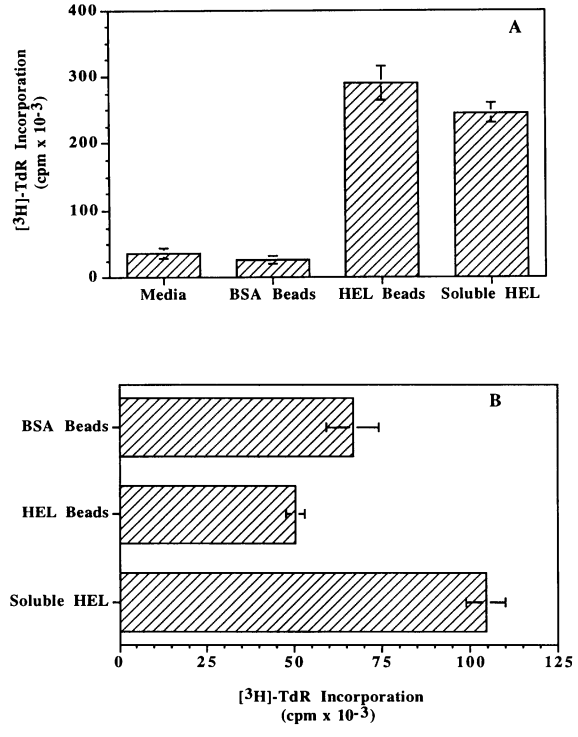

Figure 8. *Pulmonary DCs presentation of immobilized HEL* in vitro (A) *and* in vivo (B). A: *Purified pulmonary DCs from naive rats support the proliferation of HEL-immune T cells in the presence of immobilized HEL (HEL beads) inducing levels of [3H]TdR incorporation similar to comparable concentrations of soluble HEL. Immobilized irrelevant antigen (BSA-beads) induced levels of [3H]TdR incorporation comparable to media control.* B: *Purified pulmonary DCs from rats challenged with soluble HEL induced greater levels of [3H]TdR incorporation into HEL-immune T cells compared with DCs from rats challenged with HEL-beads, which induced levels comparable to controls (BSA-beads). Data are mean ± SD of triplicate samples from a representative experiment.*

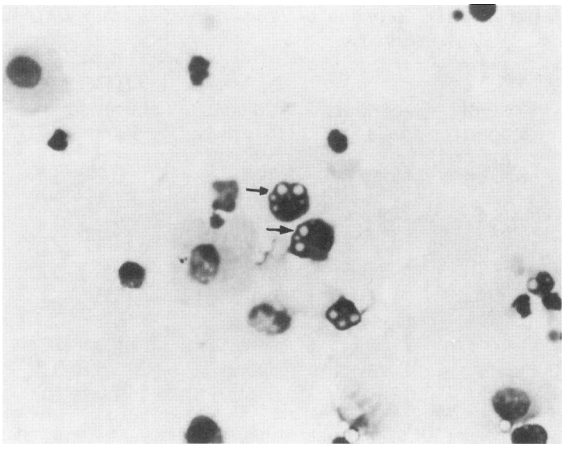

Figure 9. *Ingestion of HEL-beads by lung phagocytes after i.t. injection. Cytocentrifuge preparation of cells obtained from BAL 24 hours after i.t. challenge with HEL-beads. Note the presence of polystyrene beads in lung phagocytes* (arrows).

The earlier observations that purified pulmonary DCs can serve as APCs for HKL *in vitro* were confirmed.[12] However, the ability of pulmonary DCs to serve as APCs for HKL after an *in vivo* antigen challenge proved to be threshold dependent, so that substantial antigen presentation was seen only when the dose of HKL delivered i.t. exceeded 10^9 organisms/rat. The absence of APC activities under these conditions could not be explained by either a delayed acquisition of APC activities *in vivo* or to immunosuppressive effects within the culture system.

We speculated that the threshold dependence of the APC activities of pulmonary DCs might reflect limited antigen availability within the lung interstitium. Analysis of BAL fluid revealed large numbers of phagocytic cells, composed predominantly of PMNs that had internalized the HKL organisms. A direct analysis of lung tissue showed that the ingested organisms were predominantly within MØs distributed the along alveolar septa when the dose of injected HKL was less than 10^9 organisms/rat and that there was no evidence of HKL having penetrated the pulmonary interstitium. However, when the number of HKL administered i.t. exceeded 10^9 organisms/rat, small numbers of organisms were seen within the alveolar interstitium, where connective tissue DCs are normally located. We interpret these data to suggest that pulmonary interstitial DCs do not encounter HKL *in vivo* when these organisms are efficiently ingested by lung phagocytes. However, at higher particulate loads, antigen may escape phagocytosis and consequently become available to interstitial pulmonary DCs leading to their acquisition of APC activity.

Analysis of the draining LN cells revealed a marked increase in lymphocyte number after the secondary challenge with HKL, and specific sensitization of LN cells occurred at both low and high HKL dosages. We are currently uncertain as to how HKL is presented to T cells in the lymph node and are investigating the possibilities that MØs, PMNs, or DCs in the proximal airways may ingest HKL and subsequently participate in their transport to the draining nodes.

Despite effective sensitization of lymphocytes in the draining LN, the development of a cell-mediated immune response in the lung was limited to rats that received large numbers of HKL during the secondary challenge. We interpret this finding to suggest that the generation of a cell-mediated response in the lung may require the acquisition of APC activities by DCs in the lung interstitium.

To exclude the possibility that our findings were peculiar to the particulate antigen HKL, parallel experiments were performed with soluble HEL that had been immobilized onto polystyrene beads. After a

primary challenge, these beads were identified in phagocytes recovered from BAL. When injected i.t., immobilized HEL yielded sensitization of naive T cells in draining LNs, although a secondary challenge with these beads failed to generate a cell-mediated immune response in the lung. These findings parallel closely what had been observed for HKL. Indeed, rats sensitized with immobilized HEL and subsequently challenged with soluble HEL showed a substantial cell-mediated perivenular response in the lung. These results further support our hypothesis that interstitial lung DCs are required to mediate a pulmonary cellular response to antigen.

Other investigators have examined how the lung responds to particulate antigens. McWilliam et al[33] demonstrated a rapid recruitment of PMNs and MØs in lungs and airways within 24 hours after a challenge with heat-killed *Moraxella catarrhalis*. An increase in airway DCs was observed, and these cells were secondarily increased in the regional LN after challenge. However, anamnestic pulmonary responses to the particulate antigen were not investigated. Havenith et al[34] described the recruitment of DCs to BAL fluid after an airway challenge by particulate *Bacillus* Calmette-Guerin and demonstrated that DCs purified from BAL showed APC activity *in vitro*, whereas alveolar MØs in BAL suppressed antigen-specific T cell proliferation in a dose-dependent response.

Lipscomb[11] has outlined a three-phase model for the development of a pulmonary immune response to an inhaled antigen. During the afferent phase, antigen penetrates the bronchial epithelium and is subsequently transported to regional LNs by airway DCs. Antigen presentation to naive T cells occurs in the LN during the central processing phase. Finally, during the efferent phase, antigen-specific immune cells are recruited to sites of inflammation in the lung. Our experiments generally support this model for the development of pulmonary immunity to particulate antigens. We further propose that the compartmentalized responses in the lung and lymph node can be dissociated in the response to particulate antigens. The current studies suggest that the sensitization of T cells can occur via the afferent and central processing pathways, whereas the effector response may not occur in the lung when the availability of the particulate is limited. Although the mechanisms regulating the pulmonary effector immune response are likely multideterminant, the sequestration of antigen by phagocytes away from interstitial DCs appears to be a critical step in limiting inflammation, without sacrificing the host's capacity to develop immunity to the offending antigen. As a result, a subsequent challenge by large quantities of antigen, such as might occur for a replicating microbial pathogen, may elicit a specific pulmonary cell-mediated response that limits further antigen penetration.

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